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TITLE: The Role of Neuropeptide Y (NPY) In Uncontrolled Alcohol Drinking and Relapse Behavior Resulting From Exposure to Stressful Events

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14. ABSTRACT There is high comorbidity between post-traumatic stress disorder (PTSD) and alcohol dependence, indicating that exposure to stressful events increases the risk of alcoholism. Thus, identifying pharmacological targets with potential therapeutic value in treating PTSD-associated alcoholism is critical. An interesting candidate is neuropeptide Y (NPY). Recent evidence suggests that low NPY levels promote high alcohol consumption, and it has been established the NPY protects against stress and anxiety. The overall goal of this grant is to determine the role of NPY (and related neuropeptides) in modulating stress-induced increases of alcohol consumption using mouse models. The specific projects for the current funding year determined if A) overexpression of brain NPY with a recombinant adeno-associated virus (rAAV) vector is protective against increased alcohol consumption, and B) if mutant mice lacking normal production of NPY show enhanced sensitivity to stress-induced increases of ethanol consumption. Results indicate that overexpression of brain NPY protects against high alcohol drinking in mice, and that a lack of NPY in mutant mice increases sensitivity to stress-induced alcohol self-administration. Together, the current findings provide evidence that NPY signaling protects against the effects of stress on excessive alcohol self-administration. Thus, NPY may have therapeutic value in treating alcoholism triggered by PTSD.					
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INTRODUCTION: People who have been exposed to an extremely traumatic event, such as witnessing a death, receiving a threat of death, or experiencing a serious injury, may develop a set of symptoms known as posttraumatic stress disorder (PTSD). Events that contribute to the development of PTSD are common to individuals placed in a combat environment. Evidence suggests that there is a high comorbidity between PTSD and alcohol dependence. Given the prevalence of PTSD among veterans of war and the increased risk of alcoholism for individuals suffering from PTSD, identifying pharmacological targets with potential therapeutic value in treating PTSD-associated alcoholism may be considered of high relevance to the U.S. military. An interesting candidate is neuropeptide Y (NPY), a neurochemical that is present throughout the central nervous system. NPY is involved with a diverse set of biological functions including the integration of emotional behavior such as anxiety and depression. Interestingly, evidence suggests that low NPY levels and deletion of NPY or the NPY Y1 receptor promote high alcohol consumption. Furthermore, combat-related PTSD is associated with decreased plasma levels of NPY, and uncontrolled stress caused by exposure to military survival training results in depletion of plasma NPY levels following extended exposure. Because low NPY levels promote increased alcohol intake, reduced NPY associated with PTSD may be a factor that leaves individuals susceptible to alcoholism. **Therefore, the guiding hypothesis of the present proposal is that normal NPY signaling protects against uncontrolled alcohol drinking and relapse caused by exposure to stressful events.** To address this issue, a set of studies have been proposed using animal models of stress-induced alcohol consumption. Mutant mice lacking normal NPY signaling, and overexpression of NPY with the use of a recombinant adeno-associated virus (rAAV) vector that causes expression and constitutive secretion of NPY (rAAV-FIB-NPY), are powerful tools used in the present research. These studies will establish if normal NPY signaling protects against the effects of stress on uncontrolled alcohol drinking and relapse-like alcohol-seeking behavior.

BODY: The experiments described below fall into 2 categories: Those related to Tasks 2 and 4 of the Statement of Work, and additional experiments that were run which complement work that is outlined in this proposal. We completed Task 2, and performed experiments that provided additional insight into questions associated with Task 4 (a task that we completed in the previous funding year).

TASK 2: Determine if mutant mice lacking production of NPY Y1 receptor show enhanced sensitivity to uncontrolled alcohol self-administration caused by exposure to foot-shock stress.

To determine if the ability of stress exposure to increase alcohol drinking is modulated by the NPY Y1 receptor, we previously examined alcohol drinking by mutant mice lacking the Y1 receptor (Y1^{-/-}) and normal mice (Y1^{+/+}) following exposure to a complex stressor. The complex stressor included simultaneous exposure to a change in the mouse environment (a new housing room), exposure to daily intraperitoneal injections, and noise generated by air pumps. Each stress episode involved 16-hours per day stress exposures over a 5-day period. We used this complex stressor because we found that it was more effective at inducing stress than simple foot-shock procedures (as proposed in the grant). Data from this experiment are depicted in **Figure 1** (Arrows in **Figure 1** indicate the points at which mice were exposed to the 5-day stressor). Y1^{-/-} and Y1^{+/+} mice were given access to a 10% alcohol solution for 2-hours per day. During baseline drinking before stress exposure, there were no significant differences in alcohol intake between the Y1^{-/-} and Y1^{+/+} mice. Mice were then given 5-days of access to the complex stressor without access to alcohol (at the first arrow on graph). When alcohol was returned after the stressor, the Y1^{-/-} mice showed significantly greater levels of alcohol drinking

over 5-days relative to the normal Y1^{+/+} mice (Post-stress 1). After a second 5-day exposure to the complex stressor, there were still significant differences between the genotypes as indicated by a main effect of genotype, however the Y1^{+/+} mice began to show stress-induced increases of alcohol drinking (Post-stress 2). As we previously noted, these data show that Y1^{-/-} mice are more sensitive to stress-induced increases of alcohol drinking when compared to normal Y1^{+/+} mice. Thus, NPY is protective against excessive alcohol drinking stemming from stress exposure, and NPY modulates stress-induced alcohol intake via the Y1 receptor.

During this funding round, we finished this project by assessing the effects of our complex stressor on consumption of a 1% sucrose solution (**Figure 2**). One possibility that we needed to rule out was that exposure to the complex stressor promoted a general increase in consumption of reinforcing solutions (such as sucrose), or perhaps an increase in caloric need (since ethanol contains calories). We chose a 1% sucrose solution because we have not established that this concentration of sucrose elicits similar volumes of consumption that a 10% ethanol solution generates. Mice were first given 5-days of access to sucrose for 2-hours per day (baseline) and were then given 5-days of treatment with the complex stressor (indicated by the first arrow in **Figure 2**). After 5 more days of access to 1% sucrose, mice were given a second 5-day exposure to the complex stressor (indicated by the second arrow). Consumption data in this figure are presented as change from baseline, or sucrose consumption post-stress relative to baseline consumption (the 5-days before the first stress exposure period). As is evident in **Figure 2**, there were no obvious effects of stress on sucrose consumption, and there were no significant differences between Y1^{-/-} and Y1^{+/+} mice in the amount of sucrose consumption after each stressor. Taken together, results from the 10% ethanol consumption study and the 1% sucrose consumption study show that the effects of exposure to our complex stressor are specific to increases of ethanol consumption (the stressor did not increase sucrose intake), and that Y1^{-/-} mice are more sensitive to the effects of stress on increased ethanol drinking. These data reinforce the hypothesis that the Y1 receptor plays a protective role against stress-induced increases of ethanol intake, and thus compounds aimed at the Y1 receptor may play a therapeutic role of excessive ethanol intake and alcoholism associated with combat-related stressor (including PTSD).

TASK 4: Determine if mutant mice lacking production of NPY show enhanced sensitivity to relapse of alcohol-seeking behavior caused by exposure to foot-shock stress.

A transient increase of ethanol self-administration following interruption of ethanol access (that is, days without access to ethanol) is called the alcohol deprivation effect (ADE) and is one animal model of excessive ethanol drinking that is characteristic of relapse. With the ADE, mice show a robust increase in the rate of ethanol self-administration, relative to pre-deprivation levels, after a deprivation period. This increase is transient and usually lasts a day or two after ethanol is returned. Alcoholics show a similar rise in alcohol intake after they relapse. The neurochemical substrate that modulates this phenomenon is not well characterized. To determine if increased ethanol-reinforced lever pressing resulting from interruptions of ethanol access is modulated by NPY, NPY^{-/-} and NPY^{+/+} mice were tested using ADE procedures. Data from male and female mice are presented in **Figures 3 and 4**, respectively. Mice were trained in a 2-hour operant self-administration paradigm to lever press for 10% ethanol or water on separate response keys. Following baseline (BL), mice were given a break from the operant procedures for 4-days (*indicated by the dashed lines in the figures*), and were then retested with ethanol and water in the operant paradigm for 3 consecutive days. This interruption of operant procedures was repeated over 3 cycles (ADE 1, ADE2, and ADE 3 in each figure). Results from male mice indicate that NPY^{-/-} mice exhibited a significant increase of ethanol-reinforced lever

presses, relative to pre-deprivation BL levels, the first day after ethanol was returned following the 4-day interruption of ethanol access during ADE 1 (**Figure 3, top left panel**). Importantly, the deprivation-induced increase of lever pressing was not evident on the water-reinforced lever (**Figure 3, bottom left panel**). However, male NPY^{-/-} mice did not exhibit significant deprivation-induced increases of ethanol reinforced behavior during ADE 2 and ADE3, and male NPY^{+/+} mice never showed a significant ADE. Interestingly, deprivation-induced increases of ethanol-reinforced behavior was more robust in female NPY^{-/-} mice (**Figure 4**). After each of the 3 ADE sessions (**Figure 3, top row**), female NPY^{-/-} mice exhibited a significant increase of ethanol-reinforced lever presses the first day after ethanol was returned following the 4-day interruption of procedures (*dashed lines*) relative to pre-deprivation BL responding. In no case did NPY^{+/+} mice exhibit a significant ADE. Only at ADE 3 was there a significant deprivation-induced increase of water-reinforced responding in NPY^{-/-} mice (**Figure 4, bottom right panel**). These findings indicate that the increased motivation to perform ethanol-reinforced responding followed a period of ethanol deprivation is augmented by a lack of normal NPY production, and thus NPY protects against deprivation-induced (relapse-like) increases of ethanol consumption. Current experiments are determining if exposure to the complex stress procedure described above will augment the ADE, particularly in male NPY^{-/-} mice which showed the weaker ADE. *We will also use these procedures as part of our work towards tasks 5 and 6 (with mutant mice lacking the NPY Y1 receptor or NPY viral vectors, respectively) during the one-year budget extension that we will be requesting.*

ADDITIONAL RELATED RESEARCH: Central administration of NPY protects against binge-like ethanol drinking, while central administration of a NPY Y1 receptor antagonist increases binge-like ethanol drinking, in C57BL/6J mice.

Binge drinking is a major health concern in the United States and worldwide, and has been linked to the development of ethanol dependence. Recent reports suggest that binge drinking is a growing problem in military personnel. Binge-like ethanol consumption can be modeled in animals using the drinking-in-the-dark (DID) procedure, a paradigm which reliably elicits high voluntary ethanol consumption and pharmacologically relevant blood ethanol concentrations (BECs) of 80 mg% (0.08%, the legal limit) or higher. With this procedure, ethanol preferring C57BL/6J mice are given 2-4 hours of access to a 20% ethanol solution beginning 3-hour into their dark cycle (the time of day when mice are active). Several neurochemical systems appear to be involved in maintaining binge-like ethanol consumption in mice, and we have recently found that corticotropin-releasing factor type 1 (CRF-1) receptor antagonist protects against binge-like ethanol drinking in mice. Given the established role of NPY in modulating ethanol consumption, we recently determined if central ventricular (i.c.v.) infusion of NPY would protect against binge-like ethanol drinking. As is evident in **Figure 5**, a 3 and 10 µg infusion of NPY shortly before the 4-hours of access to 20% ethanol significantly reduced the level of ethanol consumed (**top panel**) and the BECs that were achieved by mice (**bottom panel**). Interestingly, mice treated with the control infusion (0 µg) achieved BECs well over 100 mg% (0.1%) while those treated with the 3 and 10 µg doses of NPY achieved BECs below 80 mg% (that is, below the limit that would be considered the legal level in human terms). Importantly, a 3 µg dose (i.c.v.) of NPY did not significantly alter sucrose consumption (**Figure 6**), suggesting that the effects of NPY were specific to ethanol intake. On the other hand, when given i.c.v. infusion of the NPY Y1 receptor antagonist BIBP-3226 shortly before the 4-hours of access to ethanol, the 0.01 and 0.1 µg doses significantly increased ethanol intake (**Figure 7, top panel**) and associated BECs (**Figure 7, bottom panel**). When taken together, these observations suggest that compounds which target the NPY Y1 receptor (specifically agonists) may be useful

therapeutic targets for treating human binge drinking, and thus preventing the development of alcohol dependence in at risk individuals.

KEY RESEARCH ACCOMPLISHMENTS: A list of key research accomplishments achieved during the 4th budget year of this grant are as follows:

- Established that the increased sensitivity to stress-induced increases of ethanol drinking by mutant mice lacking the NPY Y1 receptor are specific to ethanol, and thus stress exposure does not increase consumption of another reinforcing and caloric substance (i.e., sucrose).
- Established that NPY signaling is protective against relapse-like increases of ethanol consumption, as NPY^{-/-} mice show greater ethanol drinking after a period of ethanol deprivation (the ADE) relative to NPY^{+/+} mice.
- Related to the point above, female mice appear to be more sensitive to the effects of NPY deletion on deprivation-induced increases of ethanol drinking, as female NPY^{-/-} mice showed a more robust ADE than male NPY^{-/-} mice.
- Established that central administration of NPY is protective against binge-like ethanol drinking in C57BL/6J mice, and significantly reduces the BECs that are achieved. Thus, NPY may play a protective role against binge drinking in humans.
- The effects of central NPY are specific to binge-like ethanol drinking, and are not associated with alterations of sucrose consumption.
- Established that central infusion of a NPY Y1 receptor antagonist increases binge-like drinking in mice. Thus, Y1 receptor *agonists* may be useful for treating binge drinking.

REPORTABLE OUTCOMES: The following is a list of publications and published abstracts that have been supported by this grant during the 4th budget year:

PUBLICATIONS

1. Lowery, E. G., Spanos, M., Navarro, M., Lyons, A. M., Hodge, C. W., & Thiele, T. E. (under review). CRF-1 antagonist and CRF-2 agonist decrease binge-like ethanol drinking in C57BL/6J mice independent of the HPA axis.
2. Lowery, E. G. & Thiele, T. E. (under review). Pre-clinical evidence that corticotropin-releasing factor (CRF) receptor antagonists are promising targets for pharmacological treatment of alcoholism.
3. Cubero, I., Navarro, M., Carvajal, F., Lerma-Cabrera, J. M., & Thiele, T. E. (under review). Ethanol-induced increase of agouti-related protein (AgRP) immunoreactivity in the arcuate nucleus of the hypothalamus of C57BL/6J, but not 129/SvJ, inbred mice.

4. Navarro, M., Cubero, I., Ko, L., & Thiele, T. E. (2009). Deletion of agouti-related protein blunts ethanol self-administration and binge-like drinking in mice. *Genes, Brain, & Behavior*, 8, 450-458.

CONFERENCE PRESENTATIONS

1. Sprow, G. M. & Thiele, T. E. (2009). Evidence for epigenetic mechanisms in ethanol-induced locomotor sensitization in DBA/2J mice. *Society for Neuroscience Abstracts, Online*.
2. Lowery, E. G., Navarro, M., Lyons, A., & Thiele, T. E. (2009). Activation of central corticotropin-releasing factor type 2 receptors (CRF2R) attenuates high levels of ethanol consumption associated with “drinking in the dark” procedures. *Society for Neuroscience Abstracts, Online*.
3. Sprow, G. M. & Thiele, T. E. (2009). Assessment of histone acetylation in the striatum and nucleus accumbens following ethanol-induced locomotor sensitization in DBA/2J mice. *Alcoholism: Clinical & Experimental Research*, 33, 215A.
4. Navarro, M., Cubero, I., Ko, L. & Thiele, T. E. (2009). Deletion of agouti-related protein protects against binge-like ethanol drinking in C57BL/6J mice. *Alcoholism: Clinical & Experimental Research*, 33, 145A.
5. Lyons, A. M., Navarro, M., Lowery, E. G., & Thiele, T. E. (2009). The effects of stress and neuropeptide Y (NPY) signaling on binge-like ethanol drinking in C57BL/6J mice. *Alcoholism: Clinical & Experimental Research*, 33, 95A.
6. Lowery, E. G., Spanos, M., Navarro, M., Lyons, A. M., Hodge, C. W., & Thiele, T. E. (2009). Extrahypothalamic corticotropin-releasing factor maintains high levels of ethanol consumption associated with “drinking in the dark” procedures. *Alcoholism: Clinical & Experimental Research*, 33, 95A.
7. Thiele, T. E., Lowery, E. G., Sparta, D. R., & Lyons, A. M. (2009). A role for CRF-1 receptor signaling in excessive ethanol intake. *Alcoholism: Clinical & Experimental Research*, 33, 277A.
8. Hayes, D. M., McCown, T. J., Knapp, D. J., Breese, G. R., & Thiele, T. E. (2009). Neuropeptide Y in the nucleus accumbens core modulates ethanol-induced locomotor activity: A role for NPY Y2 receptors. *Alcoholism: Clinical & Experimental Research*, 33, 145A.
9. Thiele, T. E. (2009). Neuropeptide Y (NPY) Y1 and corticotropin-releasing factor (CRF)-1 receptors modulate stress-induced increases of ethanol intake in mice. Talk and poster presented at the 2009 Military Health Research Forum, Kansas City, MO.
10. Thiele, T. E., Lowery, E. G., Sparta, D. R., & Lyons, A. M. (2009). A role for CRF-1 receptor signaling in excessive ethanol intake. Talk presented at the annual meeting of the Research Society on Alcoholism, San Diego, CA.

CONCLUSIONS: We have made significant progress towards the goals of this research proposal. We have shown that NPY signaling is protective against the effects of stress on excessive ethanol drinking, and more recently that these effects of stress are specific to ethanol and do not cause increased consumption of another reinforcing and caloric substance (i.e., sucrose). We have demonstrated that NPY is protective against relapse-like increases of ethanol drinking, and that NPY appears to play a greater protective role in female mice. We are now using our paradigm to assess the effects of stress of relapse-like increases of ethanol drinking, in mutant mice lacking the Y1 receptor in mice treated with a rAAV-FIB-NPY viral vector. Additional work in our lab has shown that NPY, via the NPY Y1 receptor, also plays a protective role against binge-like drinking in mice. So what does this mean? These results have important implications for possible pharmacological medical treatment of stress-related alcoholism and alcohol relapse. Pharmacological targets aimed at the NPY system may prove to be effective in treating alcoholism resulting from exposure to traumatic events and stemming from PTSD, and may prevent relapse behavior in abstinent individuals since stress is a primary cause of relapse. Additionally, our recent results provide an exciting new possibility for NPY as a potential therapeutic treatment against binge drinking, a growing problem in both the military and civilian populations. Thus, these findings may be considered of high relevance to the U.S. military.

APPENDICES:

- Figures 1-7. In figures, * indicates significant differences between groups at the $p < 0.05$ level.
- 1 published paper that was supported by this grant.

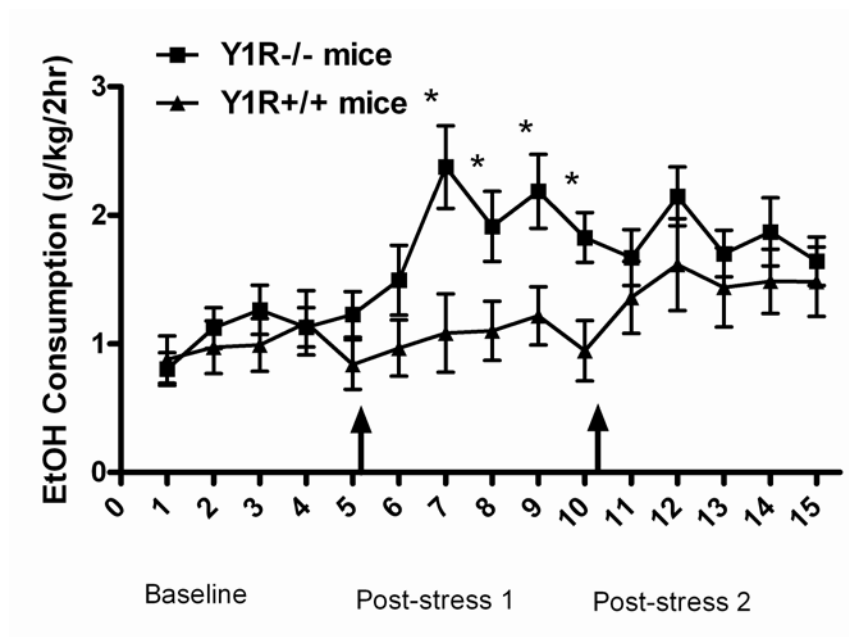


Figure 1

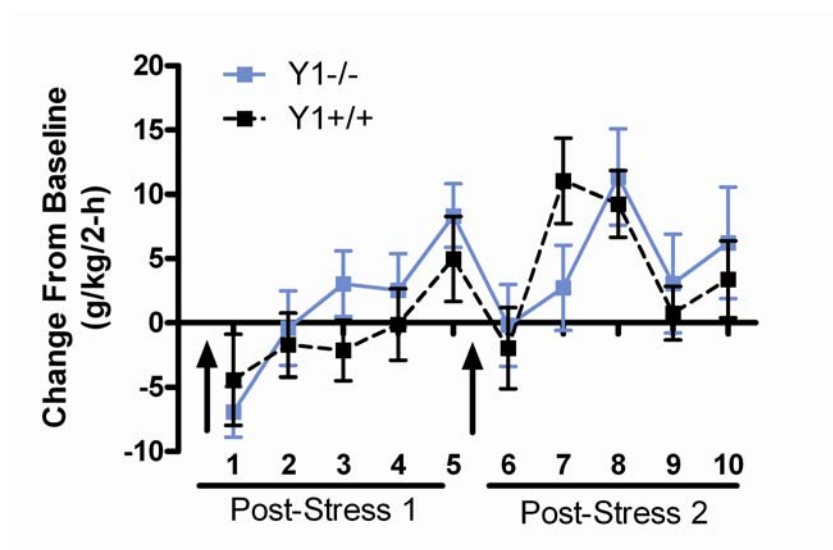


Figure 2

Male NPY-/- and NPY+/+ mice: ADE

+; NPY-/- significantly greater than BL, $p < 0.05$

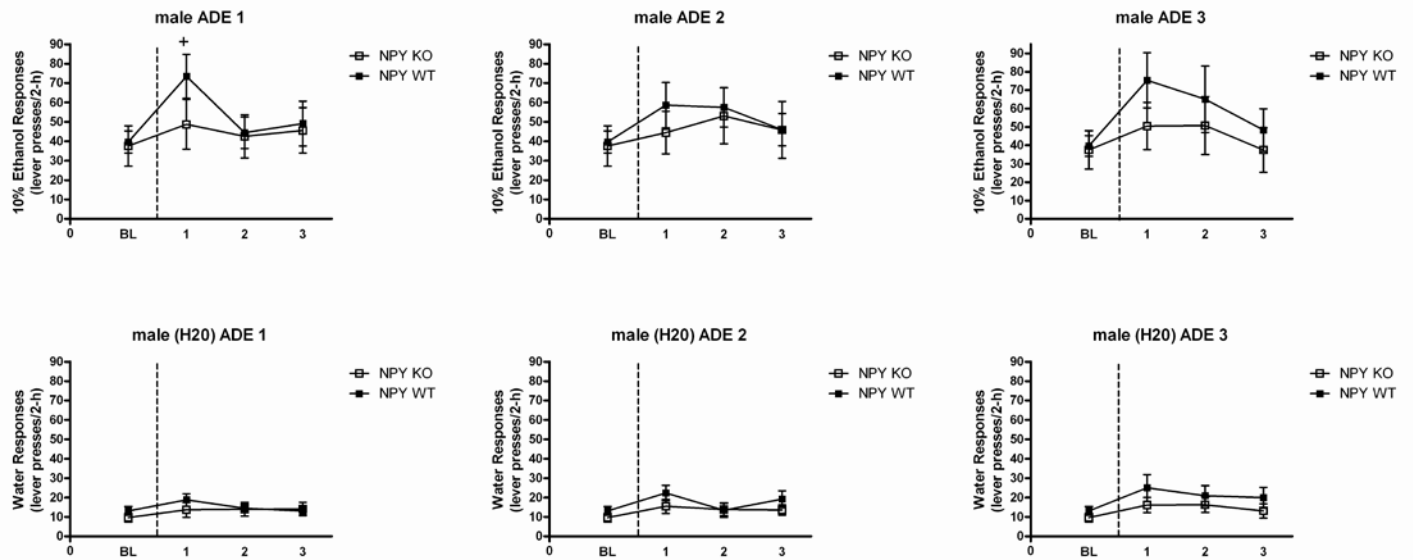


Figure 3

Female NPY-/- and NPY+/+ mice: ADE

+; NPY-/- significantly greater than BL, $p < 0.05$

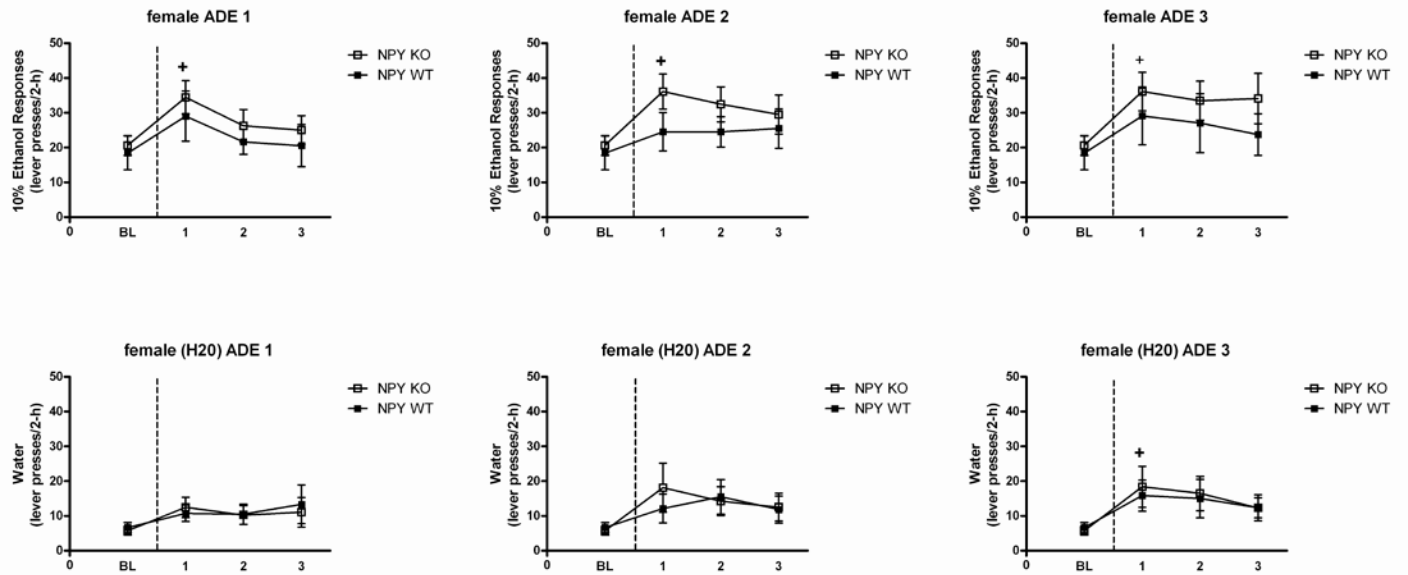


Figure 4

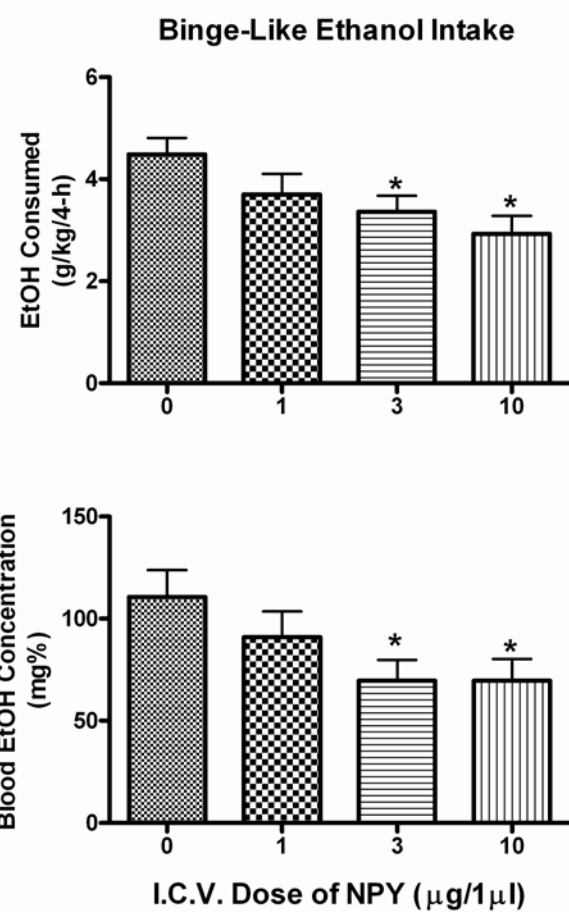


Figure 5

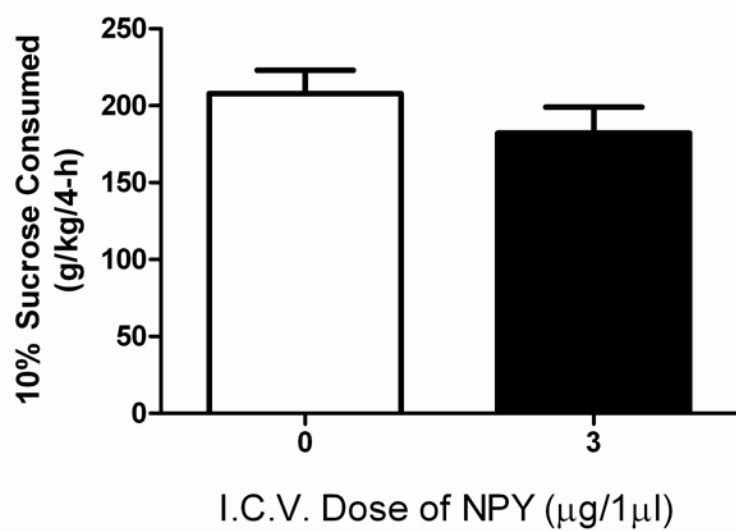


Figure 6

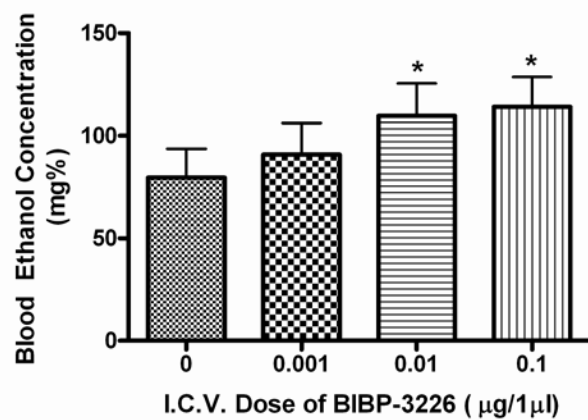
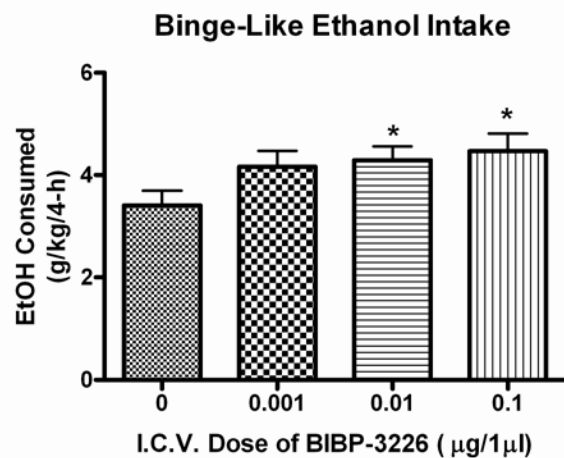


Figure 7

Deletion of agouti-related protein blunts ethanol self-administration and binge-like drinking in mice

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The melanocortin (MC) system is composed of peptides that are cleaved from the polypeptide precursor proopiomelanocortin (POMC). Recent pharmacological and genetic evidence suggests that melanocortin receptor (MCR) signaling modulates neurobiological responses to ethanol and ethanol intake. Agouti-related protein (AgRP) is synthesized by neurons in the arcuate nucleus of the hypothalamus and is a natural antagonist of MCRs. Because central administration of the functionally active AgRP fragment AgRP-(83–132) increases ethanol intake by C57BL/6 J mice, we determined if mutant mice lacking normal production of AgRP (AgRP^{−/−}) and maintained on a C57BL/6 J genetic background would show reduced self-administration of ethanol relative to littermate wild-type (AgRP^{+/+}) mice. AgRP^{−/−} mice showed reduced 8% (v/v) ethanol-reinforced lever-pressing behavior relative to AgRP^{+/+} mice in daily 2-h sessions, but normal sucrose-, saccharin- and water-reinforced lever-pressing. Similarly, AgRP^{−/−} mice showed reduced consumption of 8% ethanol in a two-bottle limited access test (2 h/day), although this effect was largely sex-dependent. Using drinking-in-the-dark (DID) procedures, AgRP^{−/−} mice showed blunted binge-like drinking of 20% (v/v) ethanol which was associated with lower blood ethanol levels (85 mg/dl) relative to AgRP^{+/+} mice (133 mg/dl) after 4 h of intake. AgRP^{−/−} mice showed normal ethanol metabolism and did not show altered sensitivity to the sedative effects of ethanol. These observations with genetically altered mice are consistent with previous pharmacological data and suggest that endogenous AgRP signaling modulates the reinforcing properties of ethanol and binge-like ethanol drinking.

Keywords: α -MSH, AgRP, binge-like drinking, C57BL/6J, ethanol, knockout, operant

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The melanocortin (MC) system is composed of peptides that are cleaved from the polypeptide precursor proopiomelanocortin (POMC). Central MC peptides are produced by neurons within the hypothalamic arcuate nucleus and the medulla (Dores *et al.* 1986; Jacobowitz & O'Donohue 1978; O'Donohue & Dorsa 1982), and include adrenocorticotrophic hormone (ACTH), α -melanocyte stimulating hormone (α -MSH), β -MSH and γ -MSH (Hadley & Haskell-Luevano 1999). Agouti-related protein (AgRP), a neuropeptide produced in the hypothalamus and secreted in the same synaptic complexes as α -MSH, functions as a natural melanocortin receptor (MCR) antagonist (Shutter *et al.* 1997).

Interestingly, α -MSH and MCRs are expressed in brain regions that have been implicated in modulating neurobiological responses to ethanol and drugs of abuse, including the striatum, nucleus accumbens (NAc), ventral tegmental area (VTA), amygdala and hippocampus (Bloch *et al.* 1979; Dube *et al.* 1978; Jacobowitz & O'Donohue 1978; O'Donohue *et al.* 1979; O'Donohue & Jacobowitz 1980; Yamazoe *et al.* 1984). Central administration of α -MSH and a non-selective MCR agonist were found to modulate dopamine signaling in the VTA and NAc (Lindblom *et al.* 2001; Lindblom *et al.* 2002a), brain regions implicated in the reinforcing properties of drugs (Ikemoto, 2007). More recently, *in vitro* single-unit recordings of VTA neurons showed that α -MSH stimulates the activity of γ -aminobutyric acid (GABA)ergic interneurons but does not influence the firing rate of dopaminergic cells (Korotkova *et al.* 2006). Because GABAergic interneurons within the VTA exert an inhibitory control of dopamine neurons (Hausser & Yung 1994; Johnson & North 1992; Paladini *et al.* 1999), α -MSH may modulate dopamine signaling indirectly via stimulation of GABAergic interneurons. Consistent with a role in modulating neurobiological responses to drugs of abuse, chronic treatment with morphine decreased MC4 receptor (MC4R) mRNA in the striatum and NAc of rats (Alvaro *et al.* 1996), and central infusion of an MCR agonist decreased the acquisition of heroin self-administration in rats (van Ree *et al.* 1981).

Ethanol also has direct effects of central MC activity. Thus, chronic exposure to ethanol significantly reduced (Navarro *et al.* 2008), while abstinence following chronic ethanol exposure increased (Kokare *et al.* 2008) endogenous α -MSH immunoreactivity in specific brain regions of Sprague-Dawley rats. Supporting a role for MCR signaling in modulating ethanol consumption, rats selectively bred for high ethanol drinking [Alko, Alcohol (AA)] were found to have low levels of MC3R in the shell of the NAc, but had high levels of MC3R and MC4R in various regions of the hypothalamus, when compared to low ethanol drinking rats

(Lindblom *et al.* 2002b). Furthermore, central infusion of the non-selective MCR agonist melanotan-II (MTII) significantly reduced voluntary ethanol drinking in AA rats (Ploj *et al.* 2002). Similarly, ventricular infusion of MTII and a selective MC4R agonist reduced ethanol drinking (Navarro *et al.* 2003; Navarro *et al.* 2005), whereas ventricular infusion of the non-selective MCR antagonist AgRP-(83–132) significantly increased ethanol drinking (Navarro *et al.* 2005), by high ethanol drinking C57BL/6 J mice. Here, we used mutant mice lacking normal production of AgRP to assess the role of endogenous AgRP in modulating neurobiological responses to ethanol.

Materials and methods

Mice

AgRP^{-/-} mice were created as described previously (Qian *et al.* 2002). Mice arrived to the laboratory on a mixed 50% 129/SvJ × 50% C57BL/6 J genetic background, and were then backcrossed with C57BL/6 J mice for eight generations. After backcrossing the mice, male and female AgRP^{+/-} mice originating from separate parents served as breeding pairs, and AgRP^{-/-} and AgRP^{+/-} littermate offspring from these breeding pairs were used in the present experiments. The genetic status of all mice was determined using polymerase chain reaction (PCR) procedures. Animals weighed approximately 20–25 g and were 2 months of age at the beginning of experiments. Mice were individually housed in polypropylene cages with corncob bedding and had *ad libitum* access to water and standard rodent chow (Tekland, Madison, WI) throughout each experiment (two food pellets were placed on the floor of the operant chambers during the 2-h session described below). The colony room was maintained at approximately 22°C with a reverse 12 h:12 h light:dark cycle with lights off at 0900 h. All procedures used in the present study were in compliance with the National Institute of Health (NIH) guidelines, and all protocols were approved by the University of North Carolina Institutional Animal Care and Use Committee. For operant and bottle-drinking studies described below, session procedures were not changed until there were at least 3 days of stable intake by the mice (i.e. no significant differences over a 3-day period).

Experiment 1: Operant self-administration of ethanol and water

Self-administration experiments were conducted in 16 modular mouse operant chambers (Med Associates, Georgia, VT) with dimensions of 21.6 × 17.8 × 12.7 cm and a stainless steel grid floor. All chambers were housed in a sound-attenuating shell with a ventilation fan. Liquid receptacles were located in the center of the right and left chamber walls and a stainless steel response lever was to the right of each receptacle. Liquid solutions (primary lever produced ethanol and a second lever produced water) were infused using 10 ml plastic syringes which were mounted on a programmable pump (PHM-100, 3.33 r.p.m.). The pump delivered 0.01 ml of solution per activation. A house light inside the operant chambers was on for the duration of the test. Data recorded during each 2-h operant session included the number of ethanol- and water-reinforced responses (bar presses), and ethanol (g/kg body weight) and water (ml/kg body weight) intake. The operant chambers were interfaced to an IBM computer and all data were automatically recorded using Med Associates software (MED-PC for Windows, Version IV). Mice were trained to lever press for 8% (v/v) ethanol reinforcement using sucrose fading procedures that we have described previously (Sparta *et al.* 2009). Briefly, over 2 days mice were placed in the operant chambers for 16 h/day and the primary lever was reinforced with 10% (w/v) sucrose solution while the second lever produced water.

Sucrose fading sessions (2 h/day) were then initiated in which ethanol (2, 4 and 8%) was added to 10% sucrose solution with a total of 4 days at each increasing concentration. Sucrose solution (2 and 5%) was then faded out with 4 days at each decreasing concentration.

Immediately after the sucrose fading procedure, mice were allowed to lever press for 8% ethanol reinforcement (primary lever) or water reinforcement (second lever) during daily 2-hour sessions beginning at 1000 h over 16 days. Sessions 17–21 involved 2-h trials that were conducted once per week, a procedure that has been reported to promote significant increases in the level of ethanol consumption by C57BL/6 J mice (Melendez *et al.* 2006). Mouse body weights were recorded immediately prior to each session to calculate ethanol consumed in grams per kilogram bodyweight or water in milliliters per kilogram bodyweight. This experiment involved male and female AgRP^{-/-} mice (*n* = 8 per sex) and male and female AgRP^{+/-} mice (*n* = 8 per sex).

Experiment 2: Operant self-administration of sucrose and saccharin solutions

This experiment was conducted to determine if altered operant self-administration of ethanol by AgRP^{-/-} mice was associated with altered self-administration of other caloric (sucrose) and non-caloric (saccharin) stimuli that can serve as reinforcers in operant self-administration procedures. The equipment and basic procedures were identical to those described in Experiment 1. Mice were first introduced to the operant procedures over 2 days (16 h/day) in which the primary lever was reinforced with 1% (w/v) sucrose solution whereas the second lever produced water. Mice then worked for 1% sucrose solution (primary lever) or water (second lever) during daily 2-h sessions beginning at 1000 h. After 4 days, the concentration of sucrose solution was increased to 3% (w/v). Following 4 days of access to 3% sucrose, mice worked for 0.01% (w/v) saccharin solution on the primary lever over 8 days and then for 0.1% (w/v) saccharin solution on the primary lever for an additional 4 days. Mouse body weights were recorded immediately prior to each session to calculate consumption of sucrose and saccharin solution in milliliters per kilogram bodyweight. This experiment involved male and female AgRP^{-/-} mice (*n* = 8 per sex) and male and female AgRP^{+/-} mice (*n* = 8 per sex).

Experiment 3: Two-bottle consumption of ethanol and water

Mice used in this experiment were the same that were involved in the ethanol operant self-administration experiment (Experiment 1). Following the final operant self-administration session, mice were tested for voluntary ethanol consumption using a homecage two-bottle choice procedure. Over an 8-day test, all mice were given two bottles on their homecage, one containing tap water and the other containing 8% (v/v) ethanol. An empty cage was used for the placement of dummy bottles (one ethanol and one water) and fluid lost from each of these bottles was subtracted off the consumption totals as a control for fluid spillage. For comparison with operant procedures, mice were given access to ethanol for 2 h beginning at 1000 h over eight daily sessions. At all other times of the day, mice had access to the water bottle only. The positions of the bottles were alternated daily to control for position preferences. Mouse body weights were recorded immediately prior to each session to calculate ethanol consumed in grams per kilogram bodyweight or water in milliliters per kilogram bodyweight. Additionally, ethanol preference ratios (volume of ethanol consumed/total volume of fluid consumed) were calculated. This experiment involved male and female AgRP^{-/-} mice (*n* = 8 per sex) and male and female AgRP^{+/-} mice (*n* = 8 per sex).

Experiment 4: Binge-like ethanol drinking and associated blood ethanol levels

This experiment involved male and female AgRP^{-/-} mice (*n* = 7 per sex) and male and female AgRP^{+/-} mice (*n* = 6 per sex). Recently,

'drinking-in-the-dark' (DID) procedures have been developed to induce excessive binge-like ethanol drinking in C57BL/6 J mice, which result in blood ethanol concentrations (BECs) reaching levels that have measurable effects on physiology and/or behavior (Rhodes *et al.* 2005, 2007). All mice in the present experiment underwent a modified DID protocol (Rhodes *et al.* 2005). Briefly, all homecage water bottles were replaced with a single bottle of 20% (v/v) ethanol, 3 h into the start of the dark phase. The 20% ethanol solution remained on the homecage for 2 h during days 1–3 of the procedures. On day 4, mice were given access to 20% ethanol, 3 h into the start of the dark cycle, for 4 h. Following the 4-h test session, tail blood (6 μ l) was collected from mice to determine BECs with an alcohol analyzer (Analox Instruments, Lunenburg, MA, USA).

A week later, the same mice were run through the same DID procedure as described above except that 1% (w/v) sucrose solution was presented to the mice during the same times that ethanol was presented above. During the following week, the mice were given access to 3% (w/v) sucrose using the same DID procedures. Mouse body weights were recorded immediately prior to each session to calculate ethanol consumed in grams per kilogram bodyweight or sucrose in milliliters per kilogram bodyweight.

Experiment 5: Ethanol-induced sedation

This experiment involved AgRP^{-/-} mice ($n = 6$ males, 7 females) and AgRP^{+/+} mice ($n = 5$ males, 6 females). Mice were transported to the testing room 45 min prior to procedures for a period of habituation. Body weights were recorded and mice were returned to their home cages. Mice received an intraperitoneal (i.p.) injection of ethanol (4.0 g/kg; 19% w/v, mixed in isotonic saline) and were returned to their home cages for observation. Upon onset of loss of

righting reflex, mice were removed from their home cages and placed on their backs in a plastic U-shaped trough. Mice were monitored and the righting reflex was considered regained when mice could right themselves onto all four paws, three times within 30 seconds. At the time mice regained the righting reflex tail blood (6 μ l) was collected from mice to determine BECs with an alcohol analyzer (Analox Instruments, Lunenburg, MA, USA).

Data analyses

All data in this report are presented as mean \pm standard error of mean (SEM). We used analyses of variance (ANOVA) to analyze all data. T-tests were used for planned comparisons (Winer *et al.* 1991). Significance was accepted at $P < 0.05$ (two-tailed).

Results

Experiment 1: Operant self-administration of ethanol and water

During the initial 2 days (sessions) of access to 10% sucrose (16 h/day), mice showed an increase in sucrose-reinforced lever-pressing between the first (130 ± 58) and second (357 ± 107) sessions which did not depend on genotype. These observations suggest that the AgRP^{-/-} and AgRP^{+/+} mice acquired sucrose-reinforced lever-pressing at a similar

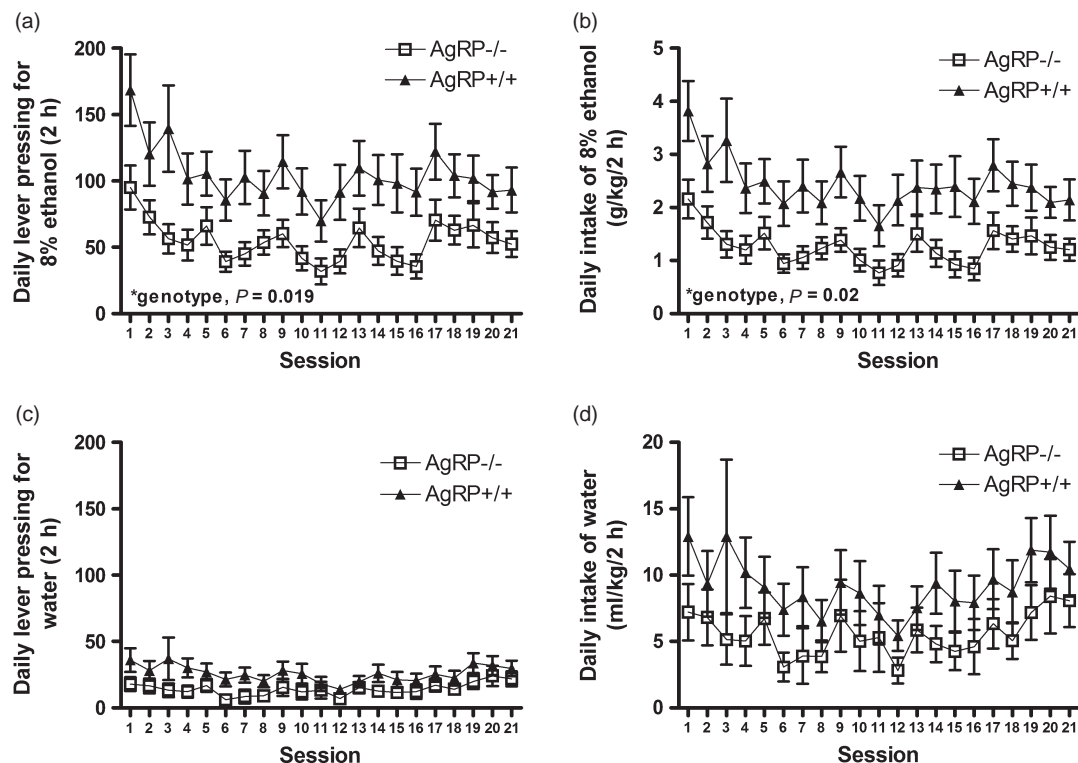


Figure 1: Deletion of AgRP blunts ethanol-reinforced lever-pressing behavior. (a) Lever responses for 8% (v/v) ethanol. (b) Consumption (g/kg) of 8% ethanol. (c) Lever responses for water. (d) Consumption (ml/kg) of water. Data were collected during daily 2-h sessions over 21 sessions. All values are means \pm SEM. *There were significant main effects of genotype for ethanol-reinforced lever-pressing and ethanol consumption data.

rate. Confirming this conclusion, a $2 \times 2 \times 2$ (genotype \times sex \times sessions) repeated-measures ANOVA performed on these data showed a main effect of session [$F(1, 28) = 37.76$, $P < 0.001$], but no other effects were statistically significant. Over 20 days of sucrose fading, AgRP^{-/-} mice exhibited significantly less ethanol/sucrose-reinforced lever-pressing (average of 126 ± 16 /day) relative to AgRP^{+/+} mice (average of 191 ± 18 /day). A $2 \times 2 \times 20$ (genotype \times sex \times sessions) repeated-measures ANOVA performed on sucrose fading data showed a significant main effect of genotype [$F(1, 28) = 7.43$, $P = 0.011$] and a significant interaction effect [$F(19, 532) = 18.62$, $P < 0.001$], confirming the above conclusion.

Ethanol- and water-reinforced lever-pressing (Fig. 1a,c) and the amount of ethanol and water consumed (Fig. 1b,d) during the 2-h operant sessions of Experiment 1 are presented in Fig. 1. Relative to AgRP^{+/+} mice, AgRP^{-/-} mice showed significantly less ethanol-reinforced lever-pressing over the 21 sessions, which was associated with significantly lower levels of ethanol consumed. A $2 \times 2 \times 21$ (genotype \times sex \times sessions) repeated-measures ANOVA performed on ethanol-reinforced lever-pressing data showed significant main effects of session [$F(20, 560) = 7.248$, $P < 0.001$] and genotype [$F(1, 28) = 6.211$, $P = 0.019$], that latter

effect confirming the overall lower level of ethanol-reinforced lever responding by AgRP^{-/-} mice. A similar repeated-measures ANOVA performed on ethanol consumption data also showed significant main effects of session [$F(20, 560) = 7.433$, $P < 0.001$] and genotype [$F(1, 28) = 6.047$, $P = 0.02$], consistent with the above conclusions. Despite a report suggesting that weekly ethanol access (rather than daily) induces escalating levels of ethanol consumption (Melendez *et al.* 2006), visual inspection of the data suggested that such an effect was not observed in the present study (Fig. 1a,b, sessions 17–21 relative to sessions 1–16). A $2 \times 2 \times 21$ (genotype \times sex \times sessions) repeated-measures ANOVA performed on water-reinforced responding and consumption data showed main effects of session [$F(20, 560) = 2.756$, $P < 0.001$] and [$F(20, 560) = 2.583$, $P < 0.001$], respectively, but both analyses failed to show significant main effects of genotype.

Experiment 2: Operant self-administration of sucrose and saccharin solutions

Sucrose- and saccharin-reinforced lever-pressing (Fig. 2a,c) and the amount of sucrose and saccharin consumed (Fig. 2b,d) during the 2-h operant sessions of Experiment

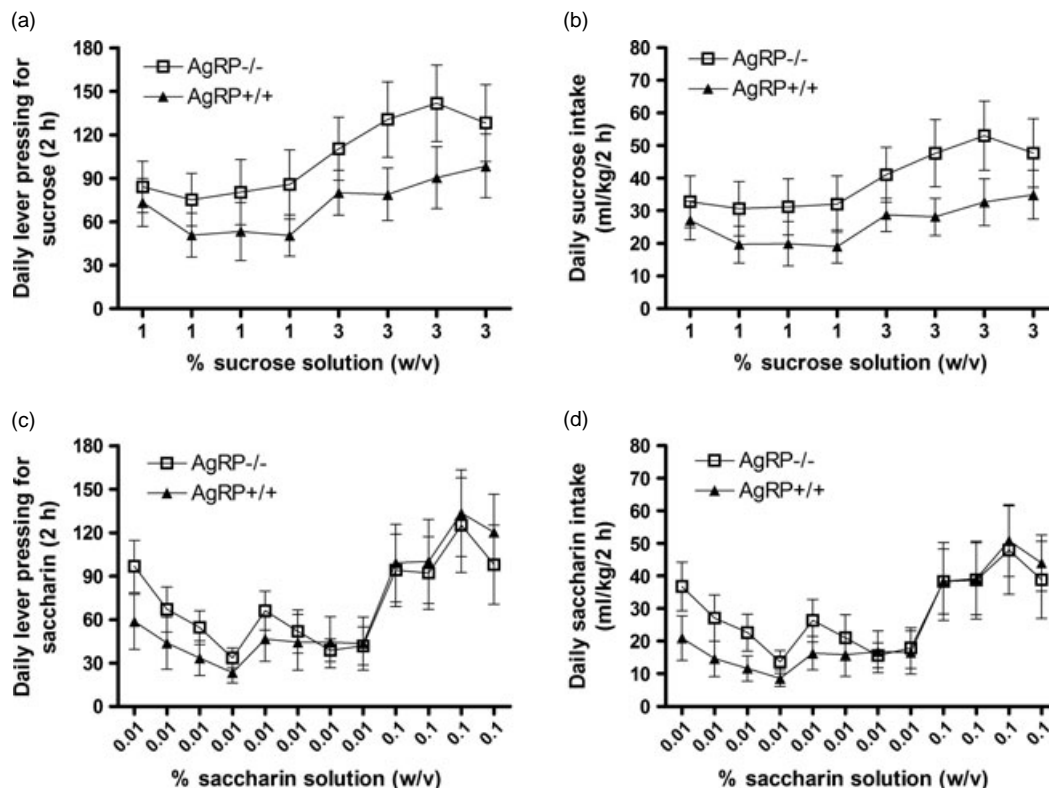


Figure 2: Deletion of AgRP does not alter sucrose- or saccharin-reinforced lever-pressing behavior. (a) Lever responses for 1 and 3% (w/v) sucrose solution. (b) Consumption (ml/kg) of 1 and 3% sucrose. (c) Lever responses for 0.01 and 0.1% (w/v) saccharin solution. (d) Consumption (ml/kg) of 0.01 and 0.1% saccharin. Data were collected during daily 2-h sessions over 8 (sucrose) or 12 (saccharin) sessions. All values are means \pm SEM. There were not significant genotype differences.

2 are presented in Fig. 2. Relative to $\text{AgRP}^{+/+}$ mice, $\text{AgRP}^{-/-}$ mice did not show significant alterations of lever-pressing for sucrose or saccharin nor did they show altered consumption of these solutions. There was a trend for increased self-administration of sucrose solution and the lower concentration of saccharin solution by $\text{AgRP}^{-/-}$ mice, opposite to the direction of genotype differences in ethanol self-administration noted earlier. Consistent with these conclusions, $2 \times 2 \times 8$ (genotype \times sex \times session) repeated-measures ANOVA performed on these data showed significant main effects of session for sucrose-reinforced responding [$F(7, 84) = 8.462, P < 0.001$], sucrose intake [$F(7, 84) = 7.735, P < 0.001$], saccharin-reinforced responding [$F(11, 132) = 12.992, P < 0.001$], and saccharin intake [$F(11, 132) = 12.825, P < 0.001$], but no other significant effects. There were no significant genotype differences in water-reinforced responding (data not shown).

Experiment 3: Two-bottle consumption of ethanol and water

A 2-h daily intake of ethanol (Fig. 3a,d) and water (Fig. 3b,e), and ethanol preference ratios (Fig. 3c,f) for male and female mice in Experiment 3 are presented in Fig. 3. A $2 \times 2 \times 8$ (genotype \times sex \times session) repeated-measures ANOVA performed on ethanol consumption data showed a significant main effect of session [$F(7, 196) = 37.904, P < 0.001$] and significant interaction effects between session and sex [$F(7, 196) = 3.989, P < 0.001$] and genotype and

sex [$F(1, 28) = 5.80, P = 0.023$]. Planned comparisons showed that female $\text{AgRP}^{-/-}$ mice drank significantly less 8% ethanol than $\text{AgRP}^{+/+}$ mice on days 1 and 4–7 of the two-bottle test. A similar repeated-measures ANOVA performed on water consumption data showed a significant main effect of session [$F(7, 196) = 9.222, P < 0.001$] and a significant session \times genotype interaction [$F(7, 196) = 2.147, P = 0.041$]. Planned comparisons showed that male $\text{AgRP}^{-/-}$ mice drank more water relative to $\text{AgRP}^{+/+}$ mice during the second session of the two-bottle test, and that female $\text{AgRP}^{-/-}$ mice drank significantly less water than $\text{AgRP}^{+/+}$ mice but only during the first daily session. Finally, a repeated-measures ANOVA performed on ethanol preference ratio data showed a significant main effect of session [$F(7, 196) = 2.797, P = 0.009$] and a significant session \times genotype interaction [$F(7, 196) = 3.987, P < 0.001$]. Planned comparisons showed that relative to $\text{AgRP}^{+/+}$ mice, male $\text{AgRP}^{-/-}$ mice exhibited a significantly lower ethanol preference ratio during session 2, and female $\text{AgRP}^{-/-}$ mice had significantly lower ethanol preference ratios on sessions 2, 4 and 7.

Experiment 4: Binge-like ethanol drinking and associated blood ethanol levels

A 2-hour consumption of 20% ethanol during the first 3 days of DID procedures are presented in Table 1. A $2 \times 2 \times 3$ (genotype \times sex \times days) repeated-measures ANOVA performed on these data showed no significant

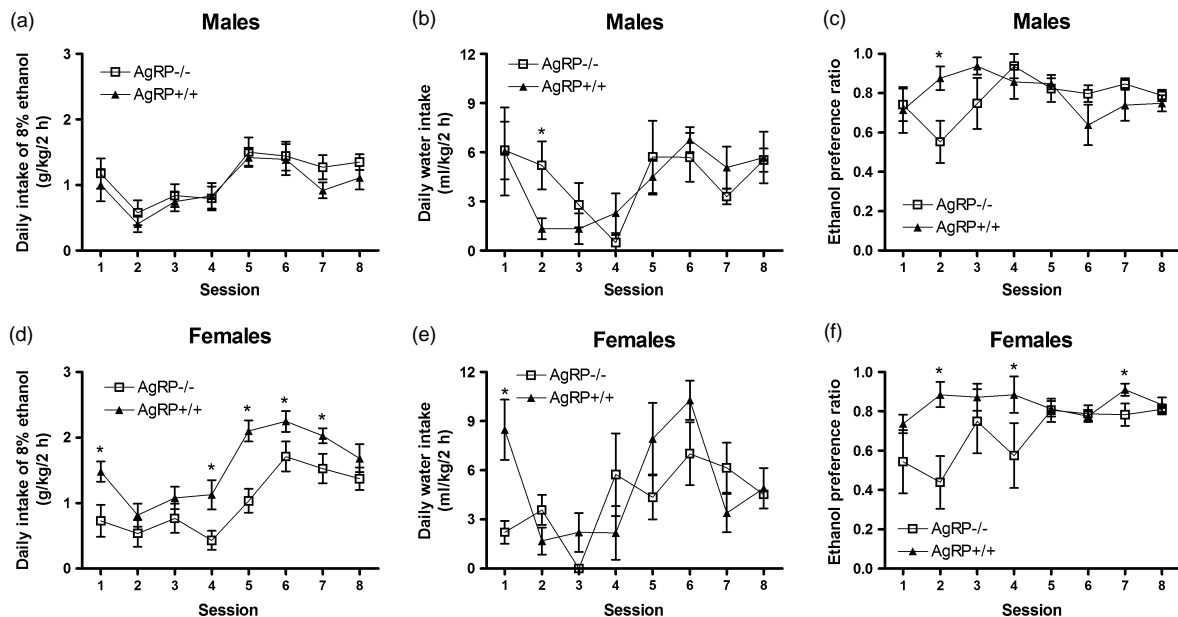


Figure 3: Deletion of AgRP attenuates ethanol consumption in female mice during two bottle-testing. (a) Consumption (g/kg) of 8% (v/v) ethanol by male mice. (b) Consumption (ml/kg) of water by male mice. (c) Ethanol preference ratios by male mice. (d) Consumption (g/kg) of 8% ethanol by female mice. (e) Consumption (ml/kg) of water by female mice. (f) Ethanol preference ratios by female mice. Data were collected during daily 2-h sessions over eight sessions. All values are means \pm SEM. * $P < 0.05$ relative to $\text{AgRP}^{+/+}$ mice.

Table 1: Ethanol consumption (g/kg/2 h) on days 1–3 (mean \pm SEM) of Experiment 4

Genotype	Day 1	Day 2	Day 3
AgRP ^{-/-}	2.79 \pm 0.23	2.95 \pm 0.31	2.58 \pm 0.27
AgRP ^{+/+}	3.33 \pm 0.31	2.56 \pm 0.33	3.24 \pm 0.30

effects. Consumption of 20% ethanol, BECs and sucrose consumption following 4 h of intake on day 4 of DID procedures are presented in Fig. 4. AgRP^{-/-} mice showed significantly lower levels of ethanol consumption (Fig. 4a) and lower associated BECs (Fig. 4b) when compared to AgRP^{+/+} mice. However, there were no genotype differences in sucrose solution consumption (Fig. 4c). A 2×2 (genotype \times sex) multi-factor ANOVA performed on ethanol consumption data showed significant main effects of genotype [$F(1, 22) = 7.549$, $P = 0.012$] and sex [$F(1, 22) = 5.646$, $P = 0.027$]. A similar multi-factor ANOVA performed on BEC data showed a significant main effect of genotype [$F(1, 22) = 4.839$, $P = 0.039$]. A $2 \times 2 \times 2$ (genotype \times sex \times concentration) repeated-measures ANOVA performed on sucrose consumption data showed a significant main effect of concentration [$F(1, 22) = 56.45$, $P < 0.001$], reflecting greater consumption of the 3% sucrose solution. No other effects were significant.

Experiment 5: Ethanol-induced sedation

Latency to regain the righting reflex following injection of a 4.0 g/kg dose of ethanol and BECs at the time of regaining the righting reflex are presented in Fig. 5. There were no significant genotype differences in time to regain the righting reflex or BECs, as 2×2 (genotype \times sex) multi-factor ANOVAs performed on these data sets failed to show any significant effects.

Discussion

Here we show that genetically altered mice lacking normal production of AgRP exhibit reduced motivation to perform ethanol-reinforced lever presses when compared to littermate wild-type control mice during daily 2-h sessions. Reduced operant self-administration of ethanol by AgRP^{-/-} mice was associated with a significant reduction in the amount of ethanol consumed relative to AgRP^{+/+} mice. Importantly, the blunted motivation to work for ethanol by AgRP^{-/-} mice was not associated with genotype differences in the acquisition of lever-pressing behavior or a general reduction in the motivation to perform reinforced behavior, as there were no significant genotype differences in the initial acquisition of sucrose-reinforced lever-pressing, and AgRP^{-/-} mice did not show significant alterations of water-, sucrose-, and saccharin-reinforced responding.

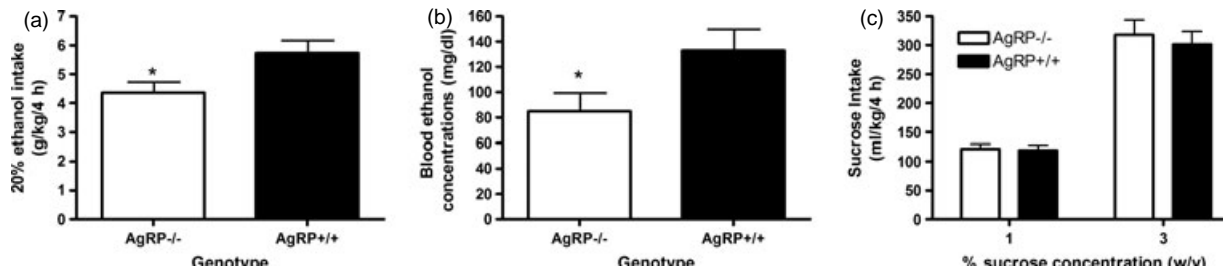


Figure 4: Mutant mice lacking AgRP are protected from binge-like ethanol drinking. (a) Consumption (g/kg) of 20% (v/v) ethanol over 4-h drinking-in-the-dark test. (b) Blood ethanol levels (mg/dl) following 4 h of ethanol consumption. (c) Consumption of 1 and 3% (w/v) sucrose solution (ml/kg) over 4 h. All values are means \pm SEM. * $P < 0.05$ relative to AgRP^{+/+} mice.

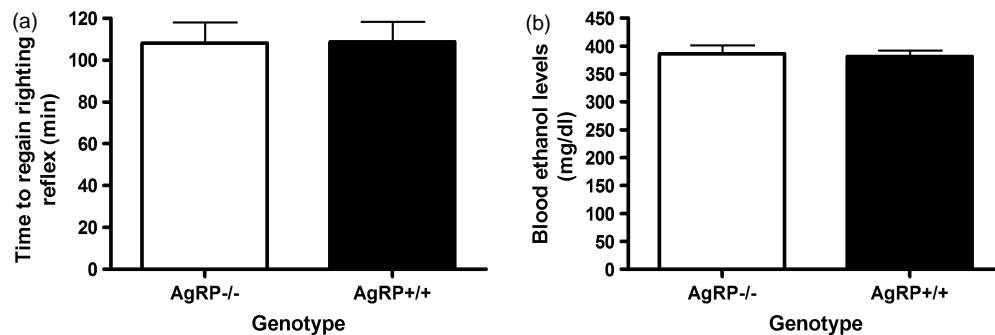


Figure 5: Mutant mice lacking AgRP show normal sensitivity to ethanol-induced sedation and normal blood ethanol levels. (a) Latency to regain the righting reflex (min) following intraperitoneal injection of a 4.0 g/kg dose of ethanol. (b) Blood ethanol levels (mg/dl) at the time of recovery of the righting reflex. All values are means \pm SEM. There were not significant genotype differences.

Thus, it is unlikely that the significant reduction of ethanol-reinforced behavior by AgRP^{-/-} mice resulted from deficits in the ability to learn or perform operant lever-pressing behavior. Interestingly, there was a non-significant trend for AgRP^{-/-} mice to exhibit increased sucrose and saccharin-reinforced lever-pressing relative to AgRP^{+/+} mice, opposite to the direction of genotype differences observed with ethanol self-administration. AgRP^{-/-} mice show normal food intake (Qian *et al.* 2002), and we show here that AgRP^{-/-} mice exhibited normal operant self-administration and consumption of sucrose, a caloric substance. Thus, it is unlikely that alterations of caloric need account for the reduced motivation to self-administer ethanol by AgRP^{-/-} mice. These data provide genetic evidence that endogenous AgRP signaling modulates the reinforcing properties of ethanol and complement our previous pharmacological data (Navarro *et al.* 2005).

Ingestive behavior (i.e. feeding and drinking) is complex and may be divided into at least two components. Appetitive behaviors are those used to locate and acquire stimuli (e.g. food and water) in the environment whereas consummatory behaviors are those used to directly consume the stimuli once they have been obtained (Samson & Hodge 1995). Operant procedures allow for the analysis of appetitive or 'seeking' responses (i.e. lever-pressing is required to gain access to the ethanol solution) while bottle-drinking procedures mainly assess consummatory behavior. Because different neuronal pathways appear to modulate appetitive versus consummatory behaviors during ethanol self-administration (Czachowski *et al.* 2001a, 2001b, 2002), we also assessed two-bottle ethanol consumption (consummatory behavior) in 2-h daily sessions for direct comparison with the ethanol-reinforced operant lever-pressing data (appetitive behavior). Similar to the operant data, AgRP^{-/-} mice showed significantly lower levels of ethanol consumption relative to AgRP^{+/+} mice during two-bottle testing, although this effect was primarily restricted to female mice. A limitation of this data set is that mice were only given access to 8% ethanol during two-bottle testing, and non-sex-dependent genotype differences might have emerged at other ethanol concentrations. In fact, AgRP^{-/-} mice also showed lower levels of 20% ethanol consumption during DID procedures which did not depend on sex. This being said, there was one day in which male AgRP^{-/-} mice showed significantly lower ethanol preference relative to AgRP^{+/+} mice during two-bottle testing. Together, the present data suggest that AgRP signaling positively modulates both appetitive and consummatory behaviors associated with ethanol self-administration.

A 'binge' is defined by the National Institute on Alcohol Abuse and Alcoholism (NIAAA) as a pattern of drinking that produces BECs greater than 80 mg/dl (NIAAA, 2004). Frequent binge drinking in humans has been linked to long-term health consequences including heart disease, high blood pressure, and type 2 diabetes (Fan *et al.* 2008). Perhaps most alarming is the finding of increased risk for developing alcohol dependence in individuals that binge drink early

in life (Hingson *et al.* 2005, 2006; Miller *et al.* 2007). DID procedures have been developed to model excessive binge-like ethanol drinking in C57BL/6 J mice (Rhodes *et al.* 2005, 2007). With these procedures, C57BL/6 J can achieve BECs of <100 mg/dl and exhibit signs of behavioral intoxication as measured by motor deficits on the rotarod and balance beam tests (Rhodes *et al.* 2005, 2007). We recently showed that binge-like ethanol drinking associated with DID procedures is unlikely motivated by caloric need, but rather by other factors such as the pharmacological post-ingestive effects of ethanol (Lyons *et al.* 2008). In the present report, we found that AgRP^{-/-} mice showed significantly reduced binge-like ethanol drinking relative to AgRP^{+/+} mice. In fact, after 4 h of ethanol consumption on day 4 of DID procedures, wild-type AgRP^{+/+} mice achieved BECs of 133 mg/dl compared to the 85-mg/dl BEC achieved by AgRP^{-/-} mice. Importantly, AgRP^{-/-} mice did not show altered consumption of 1 or 3% sucrose solution during DID procedures, and they did not exhibit alterations of recovery from ethanol-induced sleep or BECs during sedation testing. Together, these observations show that AgRP^{-/-} mice are protected from binge-like ethanol drinking, which is not related to altered caloric need, sensitivity to the intoxicating effects of ethanol, or blood ethanol clearance. Previous work has implicated opioids and dopamine (Kamdar *et al.* 2007), corticotropin releasing factor (Sparta *et al.* 2008), urocortin (Ryabinin *et al.* 2008) and glutamate (Gupta *et al.* 2008) in the modulation of binge-like ethanol drinking with DID procedures. The present observations add to this literature by showing that AgRP positively regulates binge-like drinking in C57BL/6 J mice.

It is of interest to consider the possible mechanism by which AgRP signaling modulates ethanol self-administration. Importantly, AgRP immunoreactivity has been identified in brain regions implicated in neurobiological responses to ethanol, including the VTA, NAc, amygdala, bed nucleus of the stria terminalis and lateral septum (Bagnol *et al.* 1999). Because AgRP functions as an endogenous MCR antagonist (Shutter *et al.* 1997), AgRP must influence ethanol intake by modulating MCR signaling. In fact, we and others have found that central infusion of MCR agonist attenuates ethanol consumption by high ethanol drinking rats and mice (Navarro *et al.* 2003, 2005; Ploj *et al.* 2002). On the other hand, blockade of MCR signaling by central infusion of the functionally active AgRP fragment, AgRP-(83–132), significantly increases ethanol consumption in C57BL/6 J mice (Navarro *et al.* 2005), and site-directed infusion of a selective MC4R antagonist into the NAc increased ethanol drinking in Sprague-Dawley rats (Carvajal *et al.* 2007). Thus, the MC system appears to defend against high levels of ethanol intake, while blockade of MCRs with AgRP promotes increased drinking by leaving the organism unprotected. Viewed this way, MCR signaling in AgRP^{-/-} mice is unrestrained, protecting these mice from high levels of operant ethanol self-administration and excessive binge-like ethanol drinking which are characteristic of C57BL/6 J mice.

Both *in vivo* and *in vitro* evidence shows that acute ethanol exposure increases release of endogenous opioids

from the pituitary gland and the hypothalamus (De Waele *et al.* 1992; De Waele & Gianoulakis 1993; Gianoulakis, 1990; Gianoulakis & Barcomb, 1987; Rasmussen *et al.* 1998). Non-selective opioid receptor antagonists as well as those selective for the μ or δ opioid receptors reduce ethanol consumption (Gianoulakis, 2001) and ethanol intake is reduced in μ opioid receptor knockout mice (Hall *et al.* 2001; Roberts *et al.*, 2000), demonstrating the role of opioid signaling in ethanol consumption. An interesting possibility is that AgRP and MCR signaling modulate ethanol self-administration via interactions with endogenous opioids. Consistent with the hypothesized interaction between these systems, stimulation of MCRs block the antinociceptive effects of opioids, whereas MC4R antagonists enhance opioid antinociception (Ercil *et al.* 2005; Kalange *et al.* 2007). In a similar fashion, it is interesting to speculate that blockade of MCR by AgRP may enhance endogenous opioid signaling in response to ethanol, and that stimulation of MCR signaling may blunt ethanol-induced opioid signaling. In fact, not only did an MCR agonist blunt ethanol intake in ethanol preferring rats, but also, pretreatment with the MCR agonist blocked ethanol-induced alterations of Met-enkephalin-Arg⁶Phe⁷ (MEAP) immunoreactivity in the VTA, suggesting the possibility that MCR agonism reduces ethanol intake through actions on the opioid system (Ploj *et al.* 2002).

In conclusion, here, we show that AgRP^{-/-} mice exhibit a blunted motivation to perform ethanol-reinforced behavior and do not show the robust binge-like ethanol drinking that are observed in wild-type C57BL/6 J mice. Importantly, AgRP^{-/-} mice showed unaltered water-, sucrose- and saccharin-reinforced behavior, and did not show altered ethanol metabolism or sensitivity to the sedative effects of ethanol. These observations provide direct evidence that endogenous AgRP signaling modulates the reinforcing properties of ethanol and is a positive modulator of ethanol consumption. While the precise mechanism by which AgRP modulates ethanol self-administration is unclear, an interesting possibility is that AgRP protects against the antagonistic effects of MCs on endogenous opioid activity within the mesolimbic dopamine 'reward' pathway. Opioid receptor antagonists are one of only three approved approaches for treating alcohol abuse disorder in the USA (Heilig & Egli, 2006). Thus, identifying the mechanism by which AgRP/MCR signaling and endogenous opioids interact may provide insight into new targets for treating alcoholism and improving already established therapies.

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